



**Sughrue**

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JC08 Rec'd PCT/PTO 02 MAY 2001

09/830876

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May 2, 2001

## BOX PCT

Assistant Commissioner  
for Patents  
Washington, D.C. 20231

PCT/AU99/00995-filed  
November 11, 1999

Re: Application of **John H. SKERRITT**  
entitled "**DETECTION OF PREHARVEST**  
**SPROUTING IN CEREAL GRAINS**"  
Assignee: **Quality Wheat CRC Limited**  
Our Ref: **Q-64066**

Dear Sir:

The following documents and fees are submitted herewith in connection with the above application for the purpose of entering the National stage under 35 U.S.C. § 371 and in accordance with Chapter II of the Patent Cooperation Treaty:

- ☐ an executed Declaration and Power of Attorney.
- ☐ an English translation of the International Application.
- ☒ 10 sheets of drawings.
- ☐ an English translation of Article 19 claim amendments.
- ☒ International Preliminary Examination Report (IPER).
- ☒ Statement in Support of Submission (accompanied by the Sequence Listing and a DOS version diskette containing the same).
- ☐ an executed Assignment and PTO 1595 form.
- ☒ a Form PTO-1449 listing the ISR references, and a complete copy of each reference.
- ☒ a Preliminary Amendment.

The Declaration and Power of Attorney, Assignment and Small Entity Status Declaration will be submitted at a later date.



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Assistant Commissioner  
of Patents

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It is assumed that copies of the International Application, the International Search Report, the International Preliminary Examination Report, and any Articles 19 and 34 amendments as required by § 371(c) will be supplied directly by the International Bureau. However, for the Examiner's convenience, a copy of the International Application, International Preliminary Examination Report and International Search Report are attached hereto.

Priority is claimed from November 11, 1998, based on Australian Application No. PP7058.

The Assignee for the published patent application is **QUALITY WHEAT CRC LIMITED**.

Applicant claims benefit of small entity status in accordance with 37 CFR § 1.27.

The Government filing fee is calculated as follows:

Total claims	22 - 20 =	2 x	\$9.00 =	\$18.00
Independent claims	5 - 3 =	2 x	\$40.00 =	\$80.00
Base Fee				\$500.00
<b>TOTAL FEE</b>				<b>\$598.00</b>

A check for the statutory filing fee, in the amount of \$598.00, is attached.

The Assistant Commissioner is hereby directed and authorized to charge or credit any difference or overpayment to Deposit Account No. 19-4880.

The Assistant Commissioner is also hereby authorized to charge any fees under 37 C.F.R. §§ 1.16, 1.17 and 1.492 which may be required during the entire pendency of the application to Deposit Account No. 19-4880. A duplicate copy of this transmittal letter is attached.

Respectfully submitted,

  
Gordon Kit

Registration No. 30,764

09/830876-072001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

JOHN H. SKERRITT

Appln. No.: 09/830,876

Group Art Unit: 0000

Filed: May 2, 2001

Examiner: Unknown

For: DETECTION OF PREHARVEST SPROUTING  
IN CEREAL GRAINS

STATEMENT IN SUPPORT OF SUBMISSION  
IN ACCORDANCE WITH 37 C.F.R. § 1.821

Assistant Commissioner  
of Patents  
Washington, D.C. 20231


Sir:

The following statement is provided to meet the requirements  
of 37 C.F.R. § 1.821.

I hereby state that the content of the computer readable copy  
(PatentIn Version 3.1) of the substitute Sequence Listing  
submitted in accordance with 37 C.F.R. §§ 1.821(c) and (e),  
respectively, is the same as the substitute Sequence Listing filed  
simultaneously herewith.

I hereby declare that all statements made herein of my own  
knowledge are true and that all statements made on information and  
belief are believed to be true; and further that these statements  
were made with the knowledge and that willful false statements and  
the like so made are punishable by fine or imprisonment, or both,  
under Section 1001 of Title 18 of the United States Code, and that  
such willful false statements may jeopardize the validity of this  
application or any patent issuing thereon.

7/20/01  
\_\_\_\_\_  
Date

  
\_\_\_\_\_  
Gordon Kit

09830876-072001

Applicant or Patentee: John H. SKERRITT Attorney's Docket  
Application No. 09/830876 No.: Q-64066  
Filed or Issued: May 2, 2001  
**DETECTION OF PREHARVEST SPROUTING**  
**IN CEREAL GRAINS**

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27 (c)) -- SMALL BUSINESS CONCERN**

I hereby declare that I am

- ☐ the owner of the small business concern identified below:  
☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN QUALITY WHEAT CRC LIMITED  
Riverside Corporation Park  
ADDRESS OF CONCERN North Ryde, NSW 2113, AUSTRALIA

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled: **DETECTION OF PREHARVEST SPROUTING IN CEREAL GRAIN** by inventor John H. SKERRITT

Described in ☐ the specification filed herewith  
☒ application no. 09/830876 filed May 2, 2001  
☐ patent no. \_\_\_\_\_ issued \_\_\_\_\_

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below\* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

\*NOTE: Separate verified statement are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

FULL NAME ALAN LESLIE JOHN ELWIS  
ADDRESS LOCKER BAS 1345, NORTH RYDE NSW, 1670, AUSTRALIA  
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☒ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON

SIGNING

TITLE IN ORGANIZATION

ADDRESS OF PERSON

SIGNING

Signature

ALAN ELLIS

COMPANY SECRETARY

LOCKED BAG 1345, NORTH RYDE, NSW 1670, AUSTRALIA

Date

11/5/01

OL ELL

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## PATENT APPLICATION

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

JOHN H. SKERRITT

CHAPT II filing

Appln. No.: of PCT/AU99/00995

Group Art Unit: 0000

Filed: May 2, 2001

Examiner: Unknown

For: DETECTION OF PREHARVEST SPROUTING  
IN CEREAL GRAINSPRELIMINARY AMENDMENTAssistant Commissioner  
of Patents  
Washington, D.C. 20231

Sir:

Prior to examining the above-identified application, please  
amend the application as follows.IN THE SPECIFICATION:

Page 1, before line 3, insert

--- This application is a 371 of PCT/AU99/00995, filed  
November 11, 1999. --.IN THE CLAIMS

Please amend the claims as follows:

Claim 5. (Amended) An immunoassay according to claim 1,  
wherein said first antibody or fragment thereof or said second  
antibody or fragment thereof is provided bound to a solid  
support.Claim 7. (Amended) An immunoassay according to claim 1,  
wherein binding of the second antibody or fragment thereof to

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PRELIMINARY AMENDMENT  
CHAPT II of PCT/AU99/00995

alpha-amylase is detected through the use of a readily detectable label.

Claim 9. (Amended) An immunoassay according to claim 1, wherein binding of the second antibody or fragment thereof to alpha-amylase is detected through the use of immunochromatography or agglutination.

Claim 10. (Amended) An immunoassay according to claim 1, wherein at least one of the first and second antibodies or fragments thereof is selected from monoclonal antibodies or fragments thereof and recombinant antibody fragments.

Claim 11. (Amended) An immunoassay according to claim 1, wherein the test sample is obtained from a cereal grain.

Claim 13. (Amended) An immunoassay according to claim 11, wherein the test sample is an aqueous extract from grain, grain meal or flour.

Claim 14. (Amended) An immunoassay according to claim 1, wherein said immunoassay provides for the quantitative detection of alpha-amylase by further comprising:

(iv) comparing the level of detected binding of the second antibody or fragment thereof to alpha-amylase against a suitable standard.

Claim 15. (Amended) An immunoassay according to claim 1 when used to detect whether damage in a cereal grain.

Claim 20. (Amended) A kit for preparing a two-site immunoassay for the qualitative or quantitative detection of alpha-amylase in a test sample, said kit comprising a container or solid support including a monoclonal antibody or fragment

PRELIMINARY AMENDMENT  
CHAPT II of PCT/AU99/00995


thereof, recombinant antibody or fragment thereof, recombinant antibody fragment or binding partner according to claim 16.

REMARKS

The specification has been amended to insert formal matter, the claims have amended to delete their improper multiply dependency and the Abstract has been inserted in order to make the application consistent with U.S. patent practice. Hence, the amendment of the specification and claims and the addition of the Abstract does not constitute new matter.

The Examiner is invited to contact the undersigned at his Washington telephone number on any questions which might arise.

Respectfully submitted,

  
\_\_\_\_\_  
Gordon Kit

Registration No. 30,764

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MACPEAK & SEAS, PLLC  
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Washington, D.C. 20037-3202  
Telephone: (202) 293-7060  
Facsimile: (202) 290-7860

Date: May 2, 2001



## A P P E N D I X

### Marked-up Version to Show Changes:

#### IN THE SPECIFICATION:

The specification is amended as follows:

Page 1, before line 3, the following is inserted:

--- This application is a 371 of PCT/AU99/00995, filed November 11, 1999. ---.

#### IN THE CLAIMS:

The claims are amended as follows:

Claim 5. (Amended) An immunoassay according to [any one of the preceding claims] claim 1, wherein said first antibody or fragment thereof or said second antibody or fragment thereof is provided bound to a solid support.

Claim 7. (Amended) An immunoassay according to [any one of the preceding claims] claim 1, wherein binding of the second antibody or fragment thereof to alpha-amylase is detected through the use of a readily detectable label.

Claim 9. (Amended) An immunoassay according to [any one of claims 1 to 6] claim 1, wherein binding of the second antibody or fragment thereof to alpha-amylase is detected through the use of immunochromatography or agglutination.

Claim 10. (Amended) [Am] An immunoassay according to [any one of the preceding claims] claim 1, wherein at least one of the first and second antibodies or fragments thereof is selected from monoclonal antibodies or fragments thereof and recombinant antibody fragments.

Claim 11. (Amended) An immunoassay according to [any one of the preceding claims] claim 1, wherein the test sample is obtained from a cereal grain.

Claim 13. (Amended) An immunoassay according to claim 11 [or 12], wherein the test sample is an aqueous extract from grain, grain meal or flour.

Claim 14. (Amended) An immunoassay according to [any one of the preceding claims] claim 1, wherein said immunoassay provides for the quantitative detection of alpha-amylase by further comprising: [;]

(iv) comparing the level of detected binding of the second antibody or fragment thereof to alpha-amylase against a suitable standard.

Claim 15. (Amended) An immunoassay according to [any one of the preceding claims] claim 1 when used to detect whether damage in a cereal grain.

Claim 20. (Amended) A kit for preparing a two-site immunoassay for the qualitative or quantitative detection of alpha-amylase in a test sample, said kit comprising a container or solid support including a monoclonal antibody or fragment thereof, recombinant antibody or fragment thereof, recombinant antibody fragment or binding partner according to [any one of claims 16 to 19] claim 16.

# SEQUENCE LISTING

<110> SKERRITT, John H.

<120> DETECTION OF PREHARVEST SPROUTING IN CEREAL GRAINS

<130> Q64066

<140> US 09/830,876

<141> 2001-05-02

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<151> 1999-11-11

<150> PP7058

<151> 1998-11-11

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PCT/AU99/00995

DETECTION OF PREHARVEST SPROUTING IN CEREAL GRAINSField of the Invention:

5 This invention relates to a two-site immunoassay for the qualitative or quantitative detection of alpha-amylase. The invention allows for the identification of "weather damage" in cereals (especially wheat).

Background of the Invention:

10 Weather damage or preharvest sprouting in wheat, is caused by the action of hydrolytic enzymes (amylases, proteases and lipases) in the grain endosperm. These enzymes (triggered by rain at or just before harvest), accelerate the breakdown of starch granules and protein in

15 the endosperm of germinating grain (Meredith, P.; Pomeranz, Y. Advances in Cereal Science and Technology 7 (1985) 239-299). Wheat that is weather-damaged has a significantly lower market value as a result of being rendered unsuitable for human consumption. This is

20 because the products that are made from sprouted wheat, for example breads, have grey colour, crumb texture, loaf structure and volume or in the case of noodles, poor colour and cooking qualities, due to the action of these hydrolytic enzymes, which include alpha-amylases (Orth, R.A.; Moss, H.J. Proceedings of the Fourth International

25 Conference on Pre-harvest Sprouting, D. Mares (Ed.) Westview Press, Boulder, CO., USA (1987) 167-175; Derera, N.F. (Ed.) Preharvest sprouting in cereals, CRC Press Inc., Boca Raton, FL, USA (1989)).

30 At grain delivery to the silo or elevator or during harvesting, mixing of a small quantity of damaged grain with larger amounts of sound grain can lead to downgrading of all of the grain. The necessity for accurately discriminating sprouted from sound wheat highlights the

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need for a quick, easy and reliable test for preharvest sprouting. The most common method for detecting preharvest sprouting at elevators involves the measurement of alpha-amylase activity using the "Falling Number" method, in which the consequences of enzymic hydrolysis of starch caused by amylase production is assessed as the time required for a plunger to fall through a heated slurry of wholemeal and water (Hagberg, S. Cereal Chemistry 37 (1960) 218; Perten, H. Cereal Chemistry 41 (1964) 127-140). However, the capital cost of the instrument means that it is only feasible to install them at a limited number of mills or major grain silos or elevators. The method is also relatively low in throughput and results can be affected by variation in starch pasting characteristics (D'Appolonia, B.L.; Macarthur, L.A., Pisesookbuntererng, W.; Ciacco, C.F. Cereal Chemistry 59 (1982) 254-257; Ringlund, K. Proceedings of the Third International Symposium on Pre-Harvest Sprouting in Cereals, J.E. Kruger and D.E. LaBerge (Eds.) Westview Press, Boulder, CO, USA, (1983) 111-118).

The cheaper option of visual assessment is both unreliable and not objective (Jensen, S.A.; Munck, L.; Kruger, J.E. Journal of Cereal Science 2(1984) 187-201), while other methods such as the Rapid ViscoAnalyzer (Ross, A.S.; Orth, R.A.; Wrigley, C.W. Proceedings of the Fourth International Symposium on Pre-Harvest Sprouting in Cereals, D.J. Mares (Ed.) Westview Press, Boulder, CO, USA (1987) 577-583) and Near Infrared analysis (Czuchajowska, Z.; Pomeranz, Y. Preharvest Sprouting in Cereals 1992, M.K. Walker-Simmons and J.L. Ried (Eds.) American Association of Cereal Chemists, St Paul, MN, USA (1992) 409-416), although faster, involve high capital cost. Near Infrared predictions of Falling Number are also of relatively low precision and can only discriminate



relatively large differences in Falling Number (Osborne, B.G. Journal of the Science of Food and Agriculture, 35 (1984) 106-110; Czuchajowska, Z.; Pomeranz, Y.; Preharvest Sprouting in Cereals 1992, M.K. Walker-Simmons and J.L. Ried (Eds.) American Association of Cereal Chemists, St Paul, MN, USA (1992) 409-416; Shashikumar, K.; Hazleton, J.L.; Ryu, G.H.; Walker, C.E. Cereal Foods World 38 (1993) 264-269). Direct enzyme activity assays for alpha-amylase (Barnes, W.C.; Blakeney, A.B. Die Starke 6 (1974) 193-197; McCleary, B.V.; Sheehan, H. Journal of Cereal Science 6 (1987) 237-251) are not suited for silo (elevator) or on-farm use due to a need for technical expertise and equipment such as waterbaths and filtration devices.

Immunoassays provide alternative methods for detection of preharvest sprouting through the use of antibodies that are specific for alpha-amylase isozymes. Alpha-amylases are considered to be the most appropriate targets for a test because: 1. they are relatively abundant, 2. they are synthesized early in the preharvest sprouting sequence (Corder, A.M., and Henry, R.J. Cereal Chemistry 66 (1989) 435-439), 3. they are responsible for many of the quality defects that occur when end products are prepared from sprouted wheat, and 4. the basis of the measurements in the "industry standard test" (Falling Number) is changes in the viscosity of a wholemeal-water slurry due to the presence of carbohydrate-degrading enzymes such as amylases. Earlier research has shown that specific antisera can be developed for separate- recognition of the two major groups of alpha-amylase isozymes (Daussant, J.; Renard; H.A. Cereal Research Communications 4 (1976) 201-212; Lazarus, C.M.; Baulcombe, D.C.; Martienssen, R.A. Plant Molecular Biology 5 (1985) 13-24). Immunoassay techniques have an added potential

advantage over enzyme activity assays, in that by using appropriate amylase antibodies it should be possible to specifically measure different amylase isozyme families. Immunoassay kits are generally quite robust and suitable for shipping and use in harsh environments, and can be used by individuals with little training. Such tests could not only be used by grain handlers or traders at silo (elevator) delivery of grain, but also by individual wheatgrowers. This would allow them to detect sprouting on-farm prior to harvesting in order to prevent contamination of sound wheat by sprouted grain.

The most sensitive, specific and quantitative immunoassays require the use of both a solid-phase bound antibody and a labelled detection antibody in a "two-site" assay. The detection antibody may be labelled with an enzyme, coloured particle or sol, or radioactive element or fluorophore. However, for field use without special equipment, the most useful methods are those in which the test result can be interpreted visually. The present invention relates to the development of two-site immunoassays for the qualitative or quantitative detection of alpha-amylase.

#### Disclosure of the Invention:

Thus, in a first aspect, the present invention provides a two-site immunoassay for the qualitative or quantitative detection of alpha-amylase in a test sample, said immunoassay comprising;

(i) exposing said test sample to a first ("capture") antibody or fragment thereof which specifically or preferentially binds to a first epitope on said alpha-amylase, under conditions permitting binding of said first antibody or fragment thereof to alpha-amylase if present,

(ii) subsequently exposing bound alpha-amylase, if any, to a second ("detection") antibody or fragment thereof which specifically or preferentially binds to a second epitope on said alpha-amylase that is distinct from said first epitope, under conditions permitting binding of said second antibody or fragment thereof to said bound alpha-amylase, and

(iii) detecting any binding of said second antibody or fragment thereof to said bound alpha-amylase, wherein either of said first or second epitopes is an epitope comprising one or more of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2), VNWVNKVGGS (SEQ ID NO: 3) and variants thereof showing  $\geq 80\%$ , more preferably  $\geq 90\%$ , sequence identity.

Preferred variant sequences include those that differ from the amino acid sequences IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2) and VNWVNKVGGS (SEQ ID NO: 3) in one or more conservative amino acid substitution(s). The conservative amino acid substitutions envisaged are:- G,A,V,I,L,M; D,E; N,Q; S,T; K,R,H; F,Y,W,H; and P,Na-alkalamino acids.

Preferably, either of said first or second epitopes is a conformational epitope comprising one or more of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2) and VNWVNKVGGS (SEQ ID NO: 3).

More preferably, either of said first or second epitopes is a conformational epitope comprising all of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2) and VNWVNKVGGS (SEQ ID NO: 3).

The two-site immunoassay of the present invention may be performed in accordance with any of the formats well known in the art. Particularly preferred formats include

the sandwich enzyme-linked immunosorbent assay (ELISA) and immunochromatography (IC).

In the ELISA format, the first antibody or fragment thereof is provided bound to a solid support such as a microwell plate, membrane, beads, particles or suitable sensor. It is preferable to use a blocking agent to prevent non-specific binding and to conduct the ELISA assay with washing steps as is well known in the art.

In the IC format, the second antibody or fragment thereof is provided bound to a solid support such as porous test strip. As is well known in the art, it is possible to conduct IC assays without the use of a blocking agent or washing steps.

At least one of the first and second antibodies or fragments thereof is/are preferably selected from monoclonal antibodies or fragments thereof (e.g. Fab and F(ab')<sub>2</sub>), recombinant antibodies or fragments thereof and recombinant antibody fragments (e.g. scFv). These provide significant commercial advantages over, for example, polyclonal antibodies. First, they recognise a limited number of epitopes and, for that reason, do not form aggregating complexes which can compromise ELISA or IC performance. Secondly, they are constant and reproducible reagents.

Detection of binding of the second antibody or fragment thereof may be achieved through the use of a readily detectable label such as a detectable enzyme (e.g. horseradish peroxidase or alkaline phosphatase), radioisotope (e.g. P<sup>32</sup> or S<sup>35</sup>) or luminescent or fluorescent label. Detection of binding of the second antibody might also be achieved through other means such as immunochromatography and agglutination.

The two-site immunoassay of the present invention is particularly suitable for the qualitative or quantitative

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detection of alpha-amylase in a cereal grain (e.g. wheat including bread wheat (*Triticum aestivum*), durum wheat (*Triticum turgidum* var. durum) and club wheat (*Triticum compactus*); rye (*Secale cereale*); triticale (*Triticosecale* species); barley (*Hordeum vulgare*) and related cereals (i.e. members of the *Triticeae* family), and thereby provides for the qualitative or quantitative detection of weather damage.

The test sample utilised in a two-site immunoassay for this purpose is preferably an aqueous extract from grain or, more preferably, grain meal or flour. As is described in greater detail below, alpha-amylase may be readily extracted from grain meal or flour with a dilute solution of NaCl or CaCl<sub>2</sub>.

When used for the quantitative detection of alpha-amylase in cereal grain, the two-site immunoassay further comprises a step of comparing the level of detected binding of the second antibody or fragment thereof against a suitable standard. Preferably, the level of detected binding of the second antibody or fragment thereof is positively correlated with alpha-amylase enzyme activity and negatively correlated with Falling Number.

In a second aspect, the present invention provides a monoclonal antibody or fragment thereof, recombinant antibody or fragment thereof, recombinant antibody fragment or binding partner which specifically or preferentially binds to an epitope on alpha-amylase comprising one or more of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2), VNWVNKVGGS (SEQ ID NO: 3) and variants thereof showing ≥ 80%, more preferably ≥ 90%, sequence identity.

Preferred variant sequences include those that differ from the amino acid sequences IDRLVSIRTRGQIHS (SEQ ID NO:

1), CRDDRPYADG (SEQ ID NO: 2) and VNWVNKVGGS (SEQ ID NO: 3) in one or more conservative amino acid substitution(s). The conservative amino acid substitutions envisaged are:- G,A,V,I,L,M; D,E; N,Q; S,T; K,R,H; F,Y,W,H; and P,N $\alpha$ -alkalamino acids.

Preferably, the monoclonal antibody or fragment thereof, recombinant antibody or fragment thereof, recombinant antibody fragment or binding partner of the present invention specifically or preferentially binds to a conformational epitope comprising one or more of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2) and VNWVNKVGGS (SEQ ID NO: 3).

More preferably, the monoclonal antibody or fragment thereof, recombinant antibody or fragment thereof, recombinant antibody fragment or binding partner of the present invention specifically or preferentially binds to a conformational epitope comprising all of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2) and VNWVNKVGGS (SEQ ID NO: 3).

In a third aspect, the present invention provides a kit for performing a two-site immunoassay for the qualitative or quantitative detection of alpha-amylase in a test sample, said kit comprising a container or solid support including a monoclonal antibody or fragment thereof, recombinant antibody or fragment thereof, recombinant antibody fragment or binding partner according to the second aspect.

For performing a two-site immunoassay for the qualitative or quantitative detection of alpha-amylase in a cereal grain, the kit may further comprise a container including an aqueous extraction agent for extracting alpha-amylase from grain, grain meal or flour.

Throughout this specification, unless the context requires otherwise, the term "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

The term "specifically binds" as used herein is intended to refer to binding characteristics of antibodies and fragments thereof which bind exclusively to the defined epitope on alpha-amylase or with only negligible cross-reaction with epitopes on other cereal grain substituents.

The term "preferentially binds" as used herein is intended to refer to binding characteristics of antibodies and fragments thereof which bind strongly to the defined epitope on alpha-amylase and to a lesser extent with epitopes on other cereal grain substituents.

The term "recombinant antibody", refers to an antibody that has been expressed from a host cell culture that has been transformed with an isolated, manipulated or synthesised expressible gene encoding the antibody. Methods for producing such recombinant antibodies are described in Pluckthun, A. Bio/Technology 9, 545-551 (1991).

The term "recombinant antibody fragment", refers to an antibody fragment that has been expressed from a host cell culture that has been transformed with an isolated or synthesised expressible gene encoding the antibody fragment. Examples of such recombinant antibody fragments are single chain Fv (scFv) antibody fragments. Methods for producing scFvs are described in Pluckthun, A. Bio/Technology 9, 545-551 (1991) and US Patent No. 4,946,778.

The invention is hereinafter described by reference to the accompanying figures and the following non-limiting examples describing the preparation and characterisation of suitable antibodies, their utilisation in immunoassays, and the use of immunoassays to quantify differences in alpha-amylase in wheat samples.

Brief description of the accompanying figures:

Figure 1 shows the titration of monoclonal and polyclonal antibodies to alpha-amylase using indirect ELISA. ELISA plates were coated with 1 µg purified amylase per microwell (high plus low pI isozymes from cv. Janz). Data are the mean ± SD of 3 replicates.

Figure 2 shows the performance of antibody combinations in microwell two-site ELISAs with extracts of an unsprouted (Falling Number 403) and moderately sprouted (Falling Number 187) sample. Data are shown for three capture antibodies (185612, ADL, ALI) and eight detection antibodies.

Figure 3 shows amino acid sequences with high-pI alpha-amylase clone Amy-1 13/1 recognised by seven antibodies.

Figure 4 shows the effect of antigen partial denaturation with urea on detection of high-pI alpha-amylase by three antibodies.

Figure 5 shows the discrimination of sprouted and unsprouted wheat through relationships between ELISA absorbance using a rapid tube and Falling Number for wheat samples from elevators in Queensland, with A. 59 grain samples and an assay using immobilised ALI antibody and enzyme-labelled 185612, and B. 130 grain samples and an assay using immobilised ALI antibody and enzyme-labelled ALI antibody. Samples were analyzed in duplicate in two



separate runs and raw absorbances standardized to the absorbance of a standard of Falling Number 187 units included in each run.

Figure 6 shows the relationship between immunochromatography band intensity (reflectance) and Falling Number for 13 wheat samples using antibody ADL as the capture (C) antibody plus 185612 or ADL as gold-labelled detection (D) antibody.

Figure 7 shows the within-day precision of immunochromatography tests (mean  $\pm$  standard deviations of results of 5 tests) using different antibody combinations and moderately sprouted (Falling Number 154 seconds), mildly sprouted (Falling Number 275 seconds) and unsprouted (Falling Number 382 seconds) wheat grain.

#### Examples:

#### Example 1: Production of antibodies to alpha-amylases and analysis of their specificities

##### Amylase purification

Grain from two wheat cultivars (Janz and Osprey) was germinated for 4 days at 20 °C. Germinated grain was frozen by immersion in liquid nitrogen and freeze-dried. Roots and shoots were removed and the grain was ground into wholemeal flour using a Jupiter Electric Cereal Grinder (JUPITER, Schorndorf, Germany). Alpha-amylase was extracted by stirring wholemeal flour in 20 mM sodium acetate buffer, pH 5.5 containing 1 mM CaCl<sub>2</sub> (extraction buffer) at 5 °C for 1 hour. All subsequent steps were performed at 5 °C. The extract was centrifuged (48,000 g, 30 min), and the resulting supernatant subjected to a 20 - 60 % ammonium sulfate precipitation. The material that precipitated between these salt concentrations was

redissolved and dialyzed against the extraction buffer. The dialyzed extract was filtered using a 0.8  $\mu$ m filter and purified using affinity chromatography.

A mixture of both high and low pI isozymes of alpha-amylase was isolated by  $\beta$ -cyclodextrin affinity chromatography. The affinity column (12 x 3 cm) consisted of epoxy-activated Sepharose 6B coupled to cyclohepta-amylose ( $\beta$ -cyclodextrin); Sepharose 6B was activated using 1,4-butanedioldiglycidyl ether (Sigma, St Louis, MO) according to the methods of Sundberg, L.; Porath, J. Journal of Chromatography 90 (1974) 87-98) and coupled to cycloheptaamylose using the method of Vretblad, P. FEBS Letters 47 (1974) 86-89. Alpha-amylase was eluted with 20 mM sodium acetate buffer (pH 5.5), containing 1 mM  $\text{CaCl}_2$ , 0.5 M NaCl and 8 mg/mL of cycloheptaamylose, and dialysed against 10 mM sodium acetate buffer (pH 5.5), containing 1 mM  $\text{CaCl}_2$  and reduced in volume by ultrafiltration.

Separation of high and low pI isozymes of alpha-amylase was achieved using one of three methods, either 1) ion-exchange chromatography (Lecommandeur, D. Journal of Chromatography 441 (1988) 436) using CM-Sepharose CL-6B, equilibrated with 20 mM acetate buffer, pH 4.8, containing 1 mM calcium chloride and eluted with a 0 - 0.3 M NaCl gradient, 2) preparative isoelectric focussing using a Rotofor device (Hochstrasser A.C. et al; Applied and Theoretical Electrophoresis, 1 (1991) 333-337) and pH 3-10 carrier ampholytes (Biorad, Hercules, CA, USA), or 3) immunoaffinity chromatography using a column prepared by coupling a monoclonal antibody prepared to the high pI alpha-amylase isozyme of barley (Gibson, C.E., Evans, D.E., MacLeod, L.C., Symons, M.H., Marschke, R.J., Jarratt, S., Dalton, M.R., Lance, R.C.M., Skerritt, J.H., Henry, R.J. and Fincher, G.B. Proceedings of the 44th

Australian Cereal Chemistry Conference, Royal Australian Chemical Institute, Melbourne, 44 (1994) 174-179), to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). A crude extract of amylase purified using the  
5 cycloheptaamylose method, was dialyzed against the immunoaffinity column equilibration buffer (50 mM Tris, pH 7.6 containing 5 mM  $\text{CaCl}_2$  and 0.5 M NaCl). High pI alpha-amylase was eluted with 2.5 M KSCN containing 5 mM  $\text{CaCl}_2$  and 0.5 M NaCl. The unbound fraction (thought to contain  
10 low pI amylase) was collected during loading onto the column. This fraction along with the eluted enzyme was dialyzed and concentrated as described above.

The purity of alpha-amylase prepared from crude wheat extract by affinity chromatography was shown by SDS-PAGE  
15 to contain a single band with with an approximate molecular weight of 44,000, in agreement with previous reports (Hill, R.D.; MacGregor, A.W. Advances in Cereal Science and Technology 9 (1988) pp 217-261. Isoelectric focusing of purified alpha-amylase, revealed the presence  
20 of approximately 6 to 8 bands between pH 6-7 (high pI isozymes, products of *alpha-amy 1* genes on group 6 chromosomes) and approximately four to six less strongly stained bands between pH 4.5 - 5.5 (low pI isozymes; products of *alpha-amy 2* genes on group 7 chromosomes). Two  
25 hundred grams of germinated grain yielded approximately 24 mg of a mixture of high and low pI amylase and approximately 14 mg of high pI amylase.

Production of Monoclonal and Polyclonal Antibodies to Alpha-Amylase

30 Polyclonal antisera were produced to the high pI amylase isozymes and to a mixture of both the high and low pI isozymes of the enzyme. Rabbits were given an initial injection of 1 mg protein (mixed 1:1 in Freund's Complete Adjuvant, FCA; Sigma) split between three injection sites

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in the muscles of both hind legs and subcutaneously in the neck area. Rabbits were boosted 14 and 28 days after the initial injection (500 µg protein in Freund's Incomplete Adjuvant, FICA), and blood was collected 10 days after the third booster. Sera were tested for antibodies to alpha-amylase using indirect ELISA (see below), and rabbits were boosted thereafter at monthly intervals with 500 µg protein in FICA. Antibodies were purified from rabbit sera using Gamma Bind IgG affinity chromatography.

Monoclonal antibodies (MAb) to-alpha amylase were produced by immunizing BALB/c mice with a mixture of both high and low pI isozymes of the enzyme (purified separately from both Janz and Osprey cultivars). Mice were given an initial intraperitoneal (IP) injection of 200 µg total protein mixed 1:1 in FCA. This was followed by two IP injections (in FICA) of 100 µg protein at 2-week intervals. At 4 weeks after the third injection, mice were given a final IP booster of 200 µg amylase per mouse 3 days prior to fusion. The quantity of enzyme used for immunisation was optimised in initial experiments in which 3 groups of mice (2 mice/group) were immunised initially with either 200, 40 or 10 µg total protein, and subsequently with either 100, 20 or 5 µg total protein. Blood was collected from all mice by tail bleed at 10 days after the third injection and tested for antibodies against alpha-amylase by indirect ELISA. Hybridoma production was carried out according to the general methods of Skerritt, J.H.; and Underwood, P.A. *Biochimica et Biophysica Acta* 874 (1986) 245-254, and supernatants secreted by the resulting hybridoma cells were tested for specificity to alpha-amylase using indirect ELISA (see below). Positive cell lines were subcloned cloned and

expanded by ascites, and antibodies isotyped using a Mouse-Typer Sub-isotyping Kit (BioRad, Hercules, CA). Several high-titre independent cell lines which secreted high-titer antibodies were isolated: 15764, 15689, 10185 and 10413 (all IgM, $\kappa$ ) and 15724 and 185612 were IgG<sub>1</sub>,  $\kappa$ .

Monoclonal and polyclonal antibodies were tested for specificity for alpha-amylase using semi-dry immunoblotting of SDS-PAGE gels and passive immunoblotting of isoelectric focussing (IEF) gels. SDS-PAGE gels (12 % T, 2 % C gel, run 1500 Vhr) were loaded with a crude extract of alpha amylase and electrophoresis and immunoblotting (for 4 h at 250 mA/gel) onto nitrocellulose was carried out according to Andrews, J.L.; and Skerritt, J.H. Journal of Cereal Science, 23 (1996) 61-72. IEF used 7.5% T, 3% C polyacrylamide gels (0.5 mm) with an ampholyte pH gradient of 3-10. 1 M sodium hydroxide and 1 M phosphoric acid were used as cathode and anode solutions respectively, and gels were run at 4 °C under constant power (8-10 W) for 3 hours after loading with purified  $\alpha$ -amylase. Membranes from both procedures were blocked with 3 % (w/v) bovine serum albumin (BSA) in 50 mM sodium phosphate-buffered saline, pH 7.2 (PBS), probed with MABs or PABs, and detected with alkaline phosphatase-labeled second antibodies from Promega (Madison, WI, USA).

On immunoblots of SDS-PAGE gels, both the polyclonal antiserum to high/low pI amylase and to high pI amylase primarily detected the M<sub>r</sub> 44,000 polypeptides corresponding to alpha-amylase. Weak reaction with other polypeptides in the M<sub>r</sub> 21,500 - 66,000 range (possibly fragments of alpha-amylase) was also seen. IEF immunoblots showed that the polyclonal antisera recognised both the high and low pI isozymes; the reaction of the antiserum to high pI amylases was somewhat less intense with the low pI isozymes

than the corresponding reaction of the high/low pI amylase antibody. Even though the wheat monoclonal antibodies were produced by immunizing mice with a mixture of both high and low pI isozymes of amylase, only one of the six antibodies (10581) detected both groups of isozymes; the remaining ones bound the high pI group only.

Example 2: Characterisation of antibody performance in ELISAs for alpha-amylase in wheat

Indirect and Direct ELISAs

Antibodies were purified using either Gamma Bind (Pharmacia) Protein G affinity chromatography (IgG isotypes) or ammonium sulfate precipitation (IgM isotypes). For use in direct ELISA, epitope mapping and as detecting conjugates in two-site ELISA, antibodies were coupled to horseradish peroxidase (Boehringer-Mannheim, Germany) using a method modified from Nakane, P.K.; and Kawaoi, A. (Journal of Histochemistry and Cytochemistry 22 (1974) 1084-1091). Antibodies were initially titrated by indirect and direct ELISA, before being evaluated for their ability to capture and detect alpha- amylase in sandwich ELISA. In the indirect and direct assays, 96 well plates (Nunc Maxisorp, Roskilde, Denmark) were coated for 16 h at 20 °C with 100 µL of purified alpha-amylase antigen at 1 µg/well in 50 mM carbonate buffer, pH 9.6. Wells were then washed three times with PBS - 0.05 % Tween 20 (PBST), and non-specific binding sites were blocked with 1 % BSA in PBS for 1 h at room temperature. Microwell-bound antigen was incubated for 90 min with 100 µL of antibody solution diluted in 1 % BSA in PBS and then washed three times with PBST. This was followed by a 30 min incubation with either 100 µL/well peroxidase-labelled rabbit anti-mouse or goat anti-rabbit immuno-globulins (DAKO, Glostrup, Denmark)

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5 diluted 1:2000 and 1:400 respectively in 1 % BSA in PBS. After four washes, 150  $\mu$ L substrate-chromogen (2 mM 2,2'-azino-bis-3-ethylbenzthiazoline sulfonic acid (Sigma) in 0.1 M sodium citrate buffer, pH 4.5, containing 0.003 %  $H_2O_2$  (ABTS)), was added and plates incubated for 20 min at room temperature. The enzyme reaction was terminated by the addition of 50  $\mu$ L oxalic acid (3 %, w/v), and absorbance values were measured at 414 nm. Titration against purified alpha-amylase using indirect ELISA (Figure 1) indicated that each of all the antibodies detected alpha-amylase with high sensitivity in this assay format.

#### Sandwich ELISAs

10  
15 For sandwich ELISA, plates were coated for 16 h at 20  $^{\circ}$ C with 100  $\mu$ L of either purified monoclonal antibodies or polyclonal antibodies at 1 $\mu$ g /well in 50mM carbonate buffer, pH 9.6. All subsequent steps were carried out at 20  $^{\circ}$ C. The wells were washed 3 times with PBST and non-specific binding sites blocked with 1 % BSA in PBS for 1 h. Purified alpha-amylase was serially diluted in 1 % BSA in PBST, added to the wells (100  $\mu$ L/well), and incubated for 1 h. After washing 3 times with PBST, 100  $\mu$ L of HRP-labeled monoclonal antibody or polyclonal antibody diluted in 1 % BSA in PBST was added to all wells and incubated for 30 min. The dilution of labeled antibody used had been previously determined by direct ELISA to provide an absorbance of between 1.0 and 1.5. Plates were washed as before and ABTS substrate was added to all wells. The reaction was stopped after 20 min and absorbance values were measured at 415 nm. Samples were analyzed in triplicate and the absorbance of blank wells (no addition

of alpha-amylase) was subtracted from the absorbance of each well.

All possible combinations of monoclonal and polyclonal antibodies were tested initially as capture and/or detection antibodies in the plate sandwich ELISA, using a wide range of concentrations (0.001-10 ug/mL) of purified amylase. Each polyclonal antibody functioned well as either a capture or detection antibody when used in conjunction with either the same antibody or another polyclonal antibody. Alpha-amylase was sensitively detected with a limit of detection (absorbance of 0.1 above background) of about 1 ng/mL, for either high pI amylase or high/low pI amylase. One monoclonal antibody (185612) also functioned as either a capture or detection antibody when used in conjunction with a polyclonal antibody (Figure 2). However, the other monoclonal antibodies did not function as either capture or detection reagents; i.e. there was no difference between the absorbance of wells to which amylase had been added and the blank wells which contained no enzyme. Similarly negative results were obtained when these monoclonal antibodies were immobilised through rabbit immunoglobulins to mouse immunoglobulins rather than by direct adsorption to the solid phase. Thus, although the monoclonal antibodies were specific for alpha-amylase on immunoblots and sensitively detected the enzyme in indirect ELISAs, they failed to detect amylase in a plate sandwich ELISA. Use of higher antibody coating levels (up to 10 µg/ well) and different coating buffers (e.g. phosphate-buffered saline, pH 7.2) did not effect antibody performance. A similar pattern of antibody performance was also noted in the rapid tube ELISA (Example 3) and immunochromatography formats (Example 4).



Characterisation of the epitopes recognised by anti-  
amylase antibodies

The ability of only one of the monoclonal antibodies (185612) to detect alpha-amylases in the sandwich ELISA format as either capture or detection antibody raised the possibility that it may recognise an epitope in the amylase sequence that has a distinctive sequence and other properties from the epitopes in the other monoclonal antibodies. Firstly, to identify the linear epitopes recognised by the antibodies, a series of decapeptides with pentamer overlaps was synthesised corresponding to the entire coding region of the high pI alpha-amylase from wheat (clone amy 1/13 of Baulcombe, D.C.; Huttly, A.; Martienssen, R.A.; Barker, R.A.; Jarvis, M.G. Molecular and General Genetics 209 (1987) 33-40). This is a representative of the isozyme family which is preferentially expressed in wheat grain during germination (Lazarus, C.M.; Baulcombe, D.C.; Martienssen, R.A. Plant Molecular Biology 5 (1985) 13-24; Cejudo, F.J.; Cubo, M.T.; Baulcombe, D.C. Plant Science 106 (1995) 207-213). The decapeptides were prepared by solid-phase peptide synthesis on the tips of primed pins in an 8x12 array for direct testing in microwell plates, and the binding of five monoclonal and two polyclonal antibodies analysed using indirect ELISA. Non-specific antibody binding to the pins was blocked by incubation for 1 hour at 20 °C in 2 % BSA in 0.15 M NaCl - 10 mM sodium phosphate, pH 7.2 containing 0.05 % Tween 20. The pins were transferred to wells containing peroxidase-labelled antibodies (diluted to provide a maximal absorbance of about 1.0) in 2 % BSA in 0.15 M NaCl - 10 mM sodium phosphate, pH 7.2 containing 0.05 % Tween 20 and incubated for 1 hour at 20° C with shaking, before being washed four times in 0.15 M NaCl -

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10 mM sodium phosphate, pH 7.2. Antibody binding to specific peptides was revealed by incubation in 2 mM diammonium 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) - 0.003 % (w/v) hydrogen peroxide in 50 mM sodium citrate, pH 4.5 for 40 minutes at 20 °C. Replicate assays were performed to ensure that consistent results were obtained.

The antibodies strongly bound only a small number of peptide sequences in the high pI alpha-amylase sequence (Figure 3). Antibody 185612, which functions as either capture or detection antibody in two-site assays, detected three main sequence regions. It most strongly recognised two arginine-rich sequences, which also contain other charged amino acids, especially aspartate in the case of the second amino sequence: IDRLVSIRTRGQIHS and CRDDRPYADG. In addition, a valine-rich peptide was detected: VNWVNKVGGG. A polyclonal antiserum to high pI alpha-amylase (ALI) also detected the IDRLVSIRTRGQIHS and VNWVNKVGGG sequences, as well as several other epitopes, in keeping with the polyclonal nature of the antiserum. A second polyclonal antiserum (ADL), prepared to a mixture of high and low pI alpha-amylases, also detected the VNWVNKVGGG sequence, along with several others (Figure 3). Four monoclonal antibodies which sensitively detected alpha-amylase in indirect or direct ELISA but not two-site assays, detected epitopes that were distinct from those recognised by antibody 185612 (Figure 3). In some cases the polyclonal antisera also recognised some of the same epitopes, suggesting that there are immunodominant regions in the alpha-amylase sequence. One antibody, 10413, recognised a peptide (KVGSGSPGTT) (SEQ ID NO: 5) that had a partial overlap with the valine-rich peptide recognised by antibody 185612 and the polyclonal antisera

(VNWNKVGGS). However, since 10413 did not bind to VNWNKVGGS its epitope is clearly also distinct. Thus, these results indicate that the antibodies which function in the two-site assay recognise distinct epitopes from those recognised by other antibodies specific for alpha-amylase, but which do not function in the two-site assay.

In order to assess whether there was also a difference in the conformational nature between the epitopes recognised by the antibodies which function in the two-site assay and other antibodies to alpha-amylase, effect on antibody binding of partial denaturation with urea of the alpha-amylase antigen were studied. High pI alpha-amylase was dissolved in either: 1. PBS (phosphate-buffered saline, 50 mM sodium phosphate - 150 mM NaCl, pH 7.2); 2. PBS - 1 % (w/v) dithiothreitol; 3. 8 M urea; 4. 8 M urea - 1 % (v/v) dithiothreitol, at a series of concentrations ranging from 0.01 µg/mL to 10 µg/mL. The antigens were coated onto microwell ELISA plates for 16 hours at 20-23 °C and binding to the PBS-dissolved "native" and treated amylases assessed by indirect ELISA. While each antibody detected native amylase, there was a clear difference in behaviour between different antibodies. The antibodies which functioned in two-site assays (185612 and the polyclonal antisera) bound significantly more poorly to urea-denatured amylase, while the binding of each of the four antibodies that did not function in the two-site assays was unaffected. Reduction with dithiothreitol did not similarly decrease antibody binding. Results with 185612, 15764 and 15689 are shown in Figure 4. These results provide further evidence that the epitopes recognised by antibodies which functioned in two-site assays had particular and distinct properties and that the

conformation of such epitopes was affected by partial denaturation with urea.

Example 3: Rapid tube sandwich ELISA for detection of

Alpha-Amylase in preharvest-sprouted wheat

Based on the initial performance of the antibodies in the plate sandwich ELISA, rapid tube ELISAs were developed for detection of alpha-amylase. The larger volume of the tubes makes them more suited for field use, since reagents can be added using droppers, and larger reagent volumes can be used, making the assay more acceptable to non-laboratory personnel. Sets of polystyrene test tubes (12 mm diameter x 75 mm long) were coated for 16 h at 20°C with purified capture antibody (5 µg in 500 µL 50 mM sodium carbonate buffer, pH 9.6). Tubes were washed 3 times with PBST, and non-specific binding blocked for 60 min using 1 % BSA in PBS. Large batches of tubes could be prepared by freeze drying the antibodies onto the tube surface. The conventional ELISA assay format involving sequential addition of wheat extract and enzyme conjugate, with an intermediate washing step, was simplified to provide an assay protocol involving simultaneous addition of wheat extract and enzyme (with no intermediate washing step). In this assay, antibody HRP conjugates in 1 % BSA in PBST were added (50 µL/tube) followed immediately by the addition of 500 µL of undiluted grain extract (prepared in 85 mM NaCl), and incubated for 5-10 min. The initial procedure for extraction of amylase (i.e. blending whole grain in sodium malate buffer) could be simplified by vortex mixing wholemeal flour (ground in the Jupiter mill) for 4 min in malate buffer, and the malate extraction buffer could also be replaced by a simple salt solution (Table II). The use

of any of 3 extraction solutions (85 mM or 50 mM NaCl or 50 mM NaCl plus 20 mM  $\text{CaCl}_2$ ) provided adequate extraction of enzyme and discrimination between sprouted and sound wheat. Thus an extraction buffer consisting of 85 mM NaCl (which can be made easily under field conditions by dissolving a NaCl tablet in a pre-determined volume of water) was used in subsequent experiments; this may also remove effects of variation in water quality in remote situations. Tubes were washed 3 times with 85 mM NaCl and 500  $\mu\text{L}$  of substrate-chromogen (0.6 mg/mL 3,3',5,5'-tetramethylbenzidine in 0.1 M sodium acetate, pH 5 containing hydrogen peroxide) was added to each tube. The reaction was stopped after 3-5 min by the addition of 250  $\mu\text{L}$  of 1.25 M sulfuric acid. The absorbance was measured at 450 nm using an RPA-1 Rapid Photometric analyser (Source Scientific, Garden City, CA, USA).

The tube assays were less sensitive than the plate assays with detection limits of approximately 4 ng/mL amylase, probably due to the much shorter incubation periods used. With an initial set of 10 wholemeals, mean ELISA absorbance (colour development) showed a decrease with increasing Falling Number for the tube assay in either format. The results of a comparison in which enzyme was extracted from wholemeal flour in 85 mM NaCl buffer either by intermittent vortex mixing for 4 min, or by wrist-action shaking for 15 sec, showed that hand shaking not only allowed for sufficient extraction of amylase for discrimination between sprouted and sound wheat, it also removed the need for vortex mixing, thus simplifying the tube assay procedure further.

The performance of this assay was tested with several sets of naturally-sprouted grain samples, with Falling Numbers ranging from 62 to 494. This included 56

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5 samples from Western Australia (1995 harvest), 30 samples from the 1995 harvest in New South Wales, Australia (Suneca, Hartog, Sunstate and Janz), 130 samples comprising 8 cultivars (Hartog, Cunningham, Pelsart, Banks, Sunco, Sunstate, Perouse and Janz), from Queensland, Australia (1996 harvest) and 108 samples comprising 6 cultivars (Hartog, Halberd, Sunland, Tincurrin, Matong and Vulcan) from New South Wales, Australia and subjected to controlled wetting. These varieties contained hard and soft wheat types of diverse protein contents (8-15 % protein) and end-use types. Falling Numbers for wholemeal (ground with a Falling Number 3100 mill) were determined in duplicate. The analyses were performed with the aim of establishing the relationship between Falling Number and colour development in the ELISA test, as well as establishing whether the relationships noted were substantially independent of the wheat variety analyzed.

20 The results of these analyses, shown in Figures 5A and B, indicated that relative ELISA colour development was strongly (and negatively) correlated with Falling Number for both tube assays. Wheats with Falling Numbers below 350 seconds could be discriminated from sound wheats, with decreasing Falling Numbers producing increasing assay colour.

30 The results of the ELISA also correlated with alpha-amylase activity measurements, determined using the Ceralpha assay (Megazyme International, Deltagen, Melbourne, Australia), which utilizes p-nitrophenylheptanoside as substrate (McCleary, B.V.; and Sheehan, H. Journal of Cereal Science 6 (1987) 237-251). For the 130 Queensland samples, the correlation between Ceralpha enzyme activity (units/g flour) and Falling Number was  $r = 0.93$ . Although modifications made to the

enzyme assay have increased its sensitivity, it still appeared to be less sensitive than the Falling Number test or the tube ELISA, by failing to clearly discriminate wheat samples with Falling Numbers greater than 250 seconds. Enzymes such as amylases can also be quantified directly by various enzyme assays. The tube ELISA we have developed, showed a strong negative correlation with Falling Number (for 3 diverse sets of wheats) and a positive correlation with the Ceralpha enzyme assay. The ELISA is simple and easy to use, and is reproducible over a wide range of Falling Numbers. Although the tube sandwich ELISA was less sensitive than the plate ELISA, it still had the capacity to detect Falling Numbers below 350 sec (the critical industry cut off point). Above 350 seconds, it has been shown by others that it is difficult to establish close correlations between Falling Number and alpha-amylase activity (Mares, 1989). Analysis of three sets of wheat samples from different environments demonstrated that the relationship between ELISA absorbance and Falling Number had little dependence on wheat variety. The precision of sample analysis using the field ELISA was similar to the precision of the Falling Number test.

#### Example 4: Immunochromatography assay for alpha-amylase

Immunochromatography (IC), in which bound and unbound components are separated by capillary flow rather than a washing step, potentially provides an even simpler test format than the coated-tube ELISA. Several patents and publications teach the principles of this assay format (Birnbaum, S.; Uden, C.; Magnusson, C.G.M.; Nilsson, S. Analytical Biochemistry 206 (1992) 168-171; Ching, S.; Billing, P.; Gordon, J. United States Patent No. 5,120,643, issued June 9, 1992; Lou, S.C.; Patel, C.;

Ching, S.; Gordon, J. Clinical Chemistry 39 (1993) 619-624) and it has been widely applied to the "yes-no" analysis of medical analytes such as urinary human chorionic gonadotrophin analysis in urine for pregnancy testing, and screening for infectious diseases such as malaria. The format has, however, had little application to agricultural analytes.

In the IC format used, a complex of antigen and labelled antibody is allowed to form then migrate by capillary action up a porous test strip. The complex is captured by a band of immobilised antibody on the strip. In the test format used, the wheat grain sample was ground to a fine meal, and 0.5 g meal is shaken with 6 mL of 85 mM NaCl solution in a tube provided. Two drops (60  $\mu$ L) of the grain extract are added to a sample pad where, if alpha-amylase is present, it binds to an antibody attached to visible (maroon) colloidal gold. A second antibody to alpha-amylase is immobilised as a line across the test strip. After addition of PBS - 0.05 % Tween 20 wetting agent to the sample pad, the colloidal gold and complexes of colloidal gold and alpha-amylase migrate up the test strip crossing the second antibody line across the test strip. If the sample has a Falling Number below 350 (i.e. contains significant amounts of alpha-amylase), the gold-amylase complex will be captured by the antibody on the membrane and a pink-maroon test line forms. In a negative sample, no test line forms. The card also contains a "control line" of immobilised goat anti-mouse or goat anti-rabbit immunoglobulin antibodies, which provides a positive pink-maroon result in every test.

The different antibody combinations were tested for performance in the IC assay, and only those combinations which functioned in a double-antibody sandwich ELISA (i.e.



Mab 185612 and the two polyclonal antibodies) also functioned in the double antibody IC test; antibodies 10413, 15689, 15724 and 15764 did not. Although IC is commonly regarded as a qualitative test format, we found that the intensity of the colour developed in the test line depended upon the concentration of alpha-amylase in the test sample and thus the extent of the weather damage in the sample, such that more colour indicates greater weather damage. Colourless or very pale results occurred when the sample was sound (Falling Number over 350 seconds). The level of weather damage and likely Falling Number for the sample can be determined by analysing some samples of known Falling Number and by comparing test results (Figure 6). Test results were also reproducible between and within assays (Figure 7). Results can either be read with respect to standards of known Falling Number, for example on a colour card by using a reflectometer using either white light illumination or light-emitting diode illumination. When manually reading the results of the tests, a 5 min assay time is suitable. It is possible to further decrease this time in conjunction with a reflectometer.

These assays (in the form of a simple kit) have the potential for on-farm use by individual growers allowing identification of areas of sprouting prior to harvest, thus preventing contamination of sound wheat by wheat that is weather damaged. Currently growers may harvest grain across their whole property, and tests on elevator receival are done on the whole parcel of grain. However, except in very wet harvests, the extent and presence of preharvest sprouting can vary quite markedly between and within fields, being dependant on the rate of drying of the crop after rainfall has occurred (affected by field aspect and drainage), wheat variety sown and time of

sowing. Growers usually have an intimate knowledge of the behaviour of different parts of their own property, and if they were able to test the grain from different paddocks and parts of paddocks before harvest, it should be possible to harvest the damaged grain separately from sound grain and avoid the financial losses that result from downgrading the whole crop.

The two-site immunoassays of the present invention enable the simple assessment of the level of alpha-amylase and thus the likely Falling Number of the grain sample. This is of particular advantage since the two-site immunoassays can be applied at mill or silo (elevator) receival of grain or could be used on farms with minimal equipment requirements.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Claims:

1. A two-site immunoassay for the qualitative or quantitative detection of alpha-amylase in a test sample, said immunoassay comprising;

(i) exposing said test sample to a first antibody or fragment thereof which specifically or preferentially binds to a first epitope on said alpha-amylase, under conditions permitting binding of said first antibody or fragment thereof to alpha-amylase if present,

(ii) subsequently exposing bound alpha-amylase, if any, to a second antibody or fragment thereof which specifically or preferentially binds to a second epitope on said alpha-amylase that is distinct from said first epitope, under conditions permitting binding of said second antibody or fragment thereof to said bound alpha-amylase, and

(iii) detecting any binding of said second antibody or fragment thereof to said bound alpha-amylase,

wherein either of said first or second epitopes is an epitope comprising one or more of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPFYADG (SEQ ID NO: 2), VNWVNKVGGS (SEQ ID NO: 3) and variants thereof showing  $\geq 80\%$  sequence identity.

2. An immunoassay according to claim 1, wherein either of said first or second epitopes is an epitope comprising one or more of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPFYADG (SEQ ID NO: 2), VNWVNKVGGS (SEQ ID NO: 3) and variants thereof showing  $\geq 90\%$  sequence identity.

3. An immunoassay according to claim 1, wherein either of said first or second epitopes is a conformational epitope comprising one or more of the amino acid

sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2), VNWVNVKVGGS (SEQ ID NO: 3).

4. An immunoassay according to claim 1, wherein either  
5 of said first or second epitopes is a conformational epitope comprising all of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2), VNWVNVKVGGS (SEQ ID NO: 3).
- 10 5. An immunoassay according to any one of the preceding claims, wherein said first antibody or fragment thereof or said second antibody or fragment thereof is provided bound to a solid support.
- 15 6. An immunoassay according to claim 5, wherein the solid support is selected from microwell plates, membranes, beads, particles, sensors and porous test strips.
- 20 7. An immunoassay according to any one of the preceding claims, wherein binding of the second antibody or fragment thereof to alpha-amylase is detected through the use of a readily detectable label.
- 25 8. An immunoassay according to claim 7, wherein the detectable label is selected from detectable enzymes, radioisotopes, luminescent labels and fluorescent labels.
- 30 9. An immunoassay according to any one of claims 1 to 6, wherein binding of the second antibody or fragment thereof to alpha-amylase is detected through the use of immunochromatography or agglutination.
- 35 10. An immunoassay according to any one of the preceding claims, wherein at least one of the first and second antibodies or fragments thereof is selected from

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monoclonal antibodies or fragments thereof and recombinant antibody fragments.

11. An immunoassay according to any one of the preceding claims, wherein the test sample is obtained from a cereal grain.

12. An immunoassay according to claim 11, wherein the cereal grain is selected from the group consisting of bread wheat (*Triticum aestivum*), durum wheat (*Triticum turgidum* var. durum), club wheat (*Triticum compactus*), rye (*Secale cereale*), triticale (*Triticosecale* species) and barley (*Hordeum vulgare*).

13. An immunoassay according to claim 11 or 12, wherein the test sample is an aqueous extract from grain, grain meal or flour.

14. An immunoassay according to any one of the preceding claims, wherein said immunoassay provides for the quantitative detection of alpha-amylase by further comprising;

(iv) comparing the level of detected binding of the second antibody or fragment thereof to alpha-amylase against a suitable standard.

15. An immunoassay according to any one of the preceding claims when used to detect weather damage in a cereal grain.

16. A monoclonal antibody or fragment thereof, recombinant antibody or fragment thereof, recombinant antibody fragment or binding partner which specifically or preferentially binds to an epitope on alpha-amylase comprising one or more of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2),

VNWNKVGGS (SEQ ID NO: 3) and variants thereof showing  $\geq$  80% sequence identity.

17. A monoclonal antibody or fragment thereof, recombinant antibody or fragment thereof, recombinant antibody fragment or binding partner which specifically binds to an epitope on alpha-amylase comprising one or more of the amino acid sequences: IDRLVSIIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2), VNVNWKVGGS (SEQ ID NO: 3) and variants thereof showing  $\geq 90\%$  sequence identity.

18. A monoclonal antibody or fragment thereof, recombinant antibody or fragment thereof, recombinant antibody fragment or binding partner which specifically or preferentially binds to a conformational epitope on alpha-amylase comprising one or more of the amino acid sequences; IDRLVSIIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2), VNVWNVKVGGS (SEQ ID NO: 3).

19. A monoclonal antibody or fragment thereof, recombinant antibody or fragment thereof, recombinant antibody fragment or binding partner which specifically or preferentially binds to conformational epitope on alpha-amyrase comprising all of the amino acid sequences; IDRLVSIIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2), VNVVNKVGGS (SEQ ID NO: 3).

20. A kit for performing a two-site immunoassay for the qualitative or quantitative detection of alpha-amylase in a test sample, said kit comprising a container or solid support including a monoclonal antibody or fragment thereof, recombinant antibody or fragment thereof, recombinant antibody fragment or binding partner according to any one of claims 16 to 19.

21. A kit according to claim 20, further comprising a container including an aqueous extraction agent for extracting alpha-amylase from grain, grain meal or flour.
- 5 22. A kit according to claim 20, wherein the extraction agent is aqueous NaCl or CaCl<sub>2</sub>.

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A B S T R A C T

A two-site immunoassay for the qualitative or quantitative detection of alpha-amylase in a test sample, said immunoassay comprising: (1) exposing said test sample to a first ("capture") antibody or fragment thereof which specifically or preferentially binds to a first epitope on said alpha-amylase, under conditions permitting binding of said first antibody or fragment thereof to alpha-amylase if present, (ii) subsequently exposing bound alpha-amylase, if any, to a second ("detection") antibody or fragment thereof which specifically or preferentially binds to a second epitope on said alpha-amylase that is distinct from said first epitope, under conditions permitting binding of said second antibody or fragment thereof to said bound alpha-amylase, and (iii) detecting any binding of said second antibody or fragment thereof to said bound alpha-amylase, wherein either of said first or second epitopes is an epitope comprising one or more of the amino acid sequences: IDRLVSIRTRGQIHS (SEQ ID NO:1), CRDDRPYADG (SEQ ID NO:2), VNWVNKVGGS (SEQ ID NO:3) and variants thereof showing  $\geq 80\%$ , more preferably  $\geq 90\%$ , sequence identity. The immunoassay is useful for detecting weather damage (i.e., preharvest sprouting) in cereal grain.

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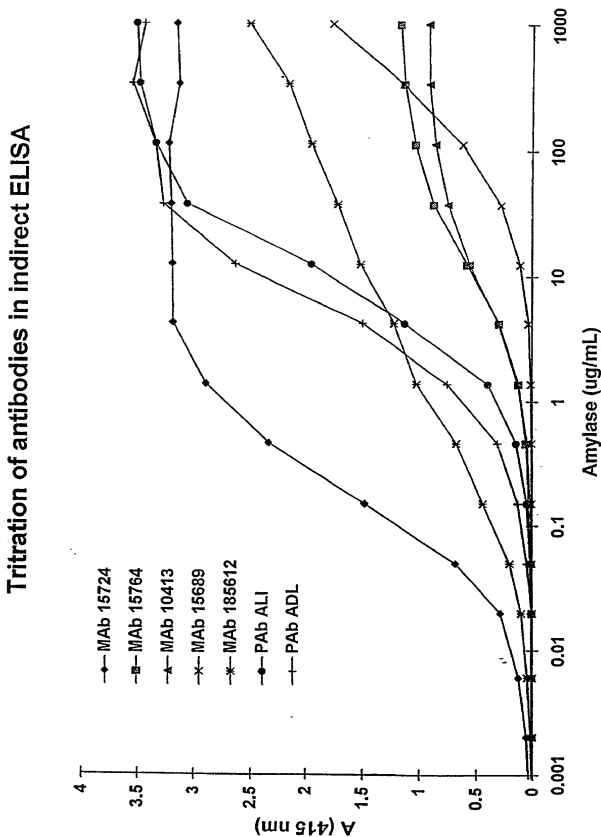


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FIGURE 1

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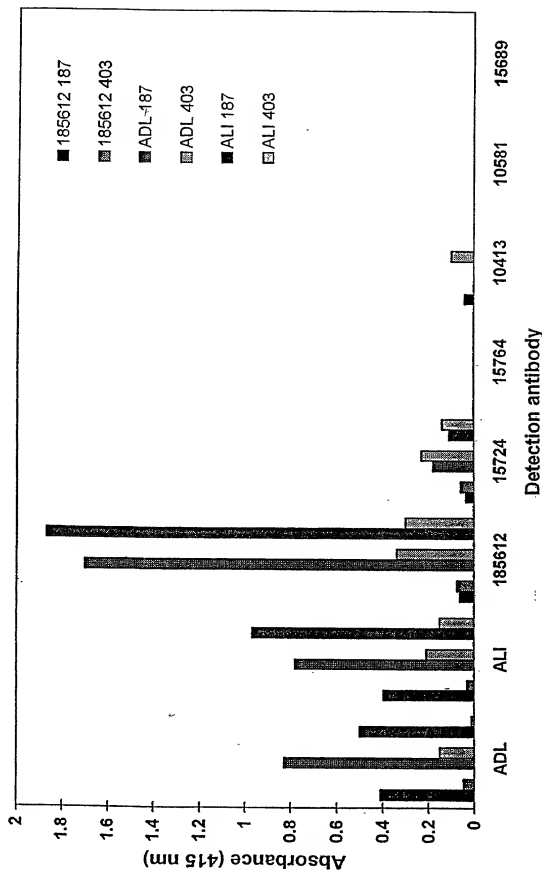
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FIGURE 2

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Performance of antibody combinations in two-site ELISAs



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FIGURE 3

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100220\*9/80E860

any 1/13	MASKHLSLEVLGLSLASQVLFQGFNWSKXHGWNFLMGKVDIAAGVTHVWLPSPQSSEQYMPGRLYDLDAASKYGNKQKSLIGALH
185612	ADL
10413	ALI
15689	15724
15764	15764
any 1/13	GKGVKALADI VNHRTAERKDRGRIYCIFEGGTFDARLDWGPMMICRDRPYADTGNPTDCAFGAAPPDIDHINPVCKELVELLNIWRTDIFGDFHRE
185612	ADL
10413	ALI
15689	15724
15764	15764
any 1/13	DFANGYADVAKTYVDORSESAFAVAEIWTSLAYGGDKPNLQDHRQELVWNVKVGSGPGTTFTTYKGIINVAVEGELWRLAGTDGKAFEMIGWPP
185612	ADL
10413	ALI
15689	15724
15764	15764
any 1/13	AKAVTVDNHDTGTCORWFFPSDRVMGVAYILTHRGPCCI FYDHFDFWGLKEIDRIIVSIRTRQGIHSESKIQIIPADADLYIAEIDGKIVTKLGPRI
185612	ADL
10413	ALI
15689	15764
any 1/13	DVGHLPGLKVAHAKDYAIWEKI (SEQ ID NO: 4)
185612	ADL
10413	ALI
15689	15764

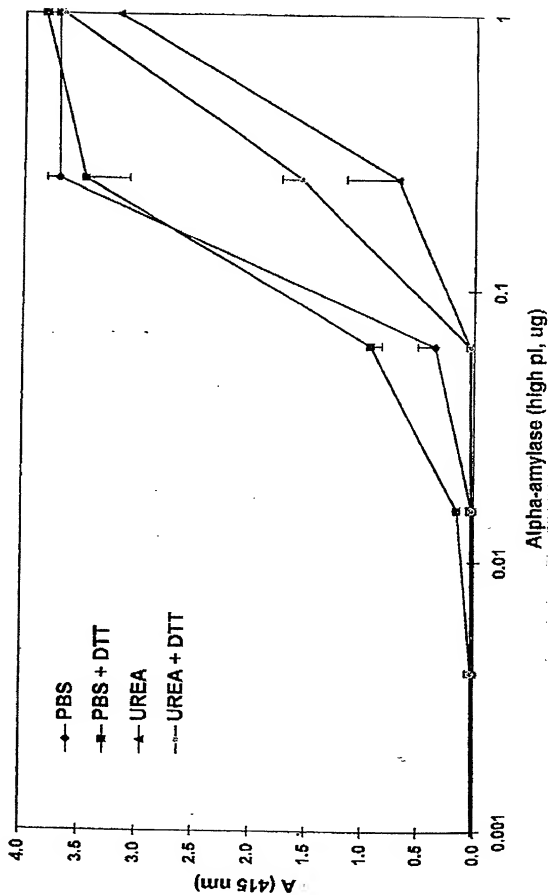
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FIGURE 4a

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# Antibody 185612: effect of antigen denaturation



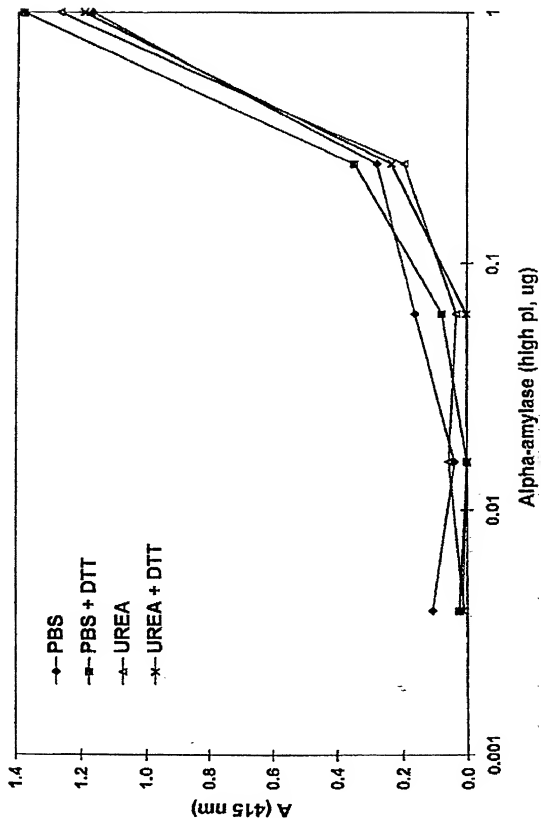
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FIGURE 4b

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Antibody 15689: effect of antigen denaturation



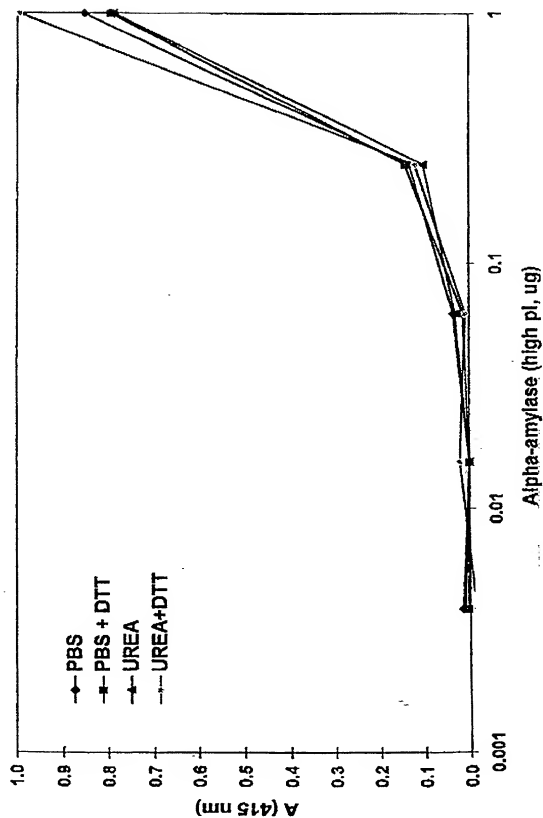
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FIGURE 4c

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Antibody 15764: effect of antigen denaturation



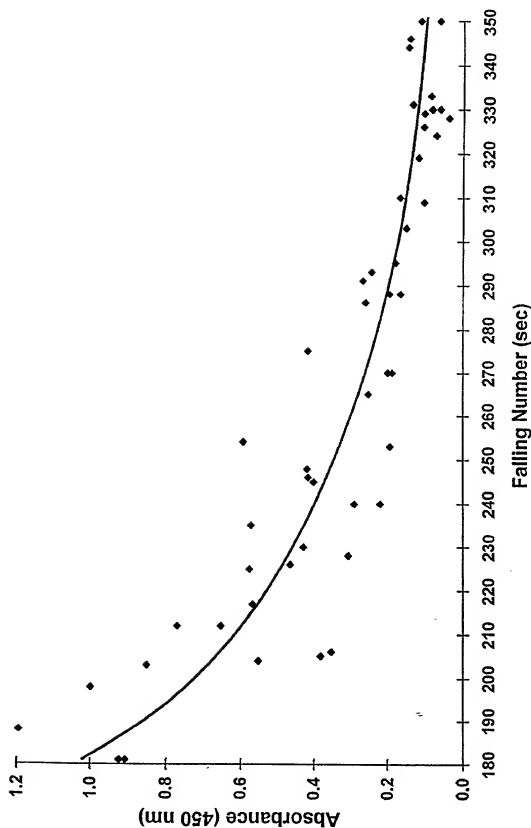
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FIGURE 5a

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Discrimination of sprouted and unsprouted wheat using  
antibody 185612 in a rapid tube ELISA



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FIGURE 5b

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Discrimination of sprouted and unsprouted wheat using  
antibody ALI in a rapid tube ELISA

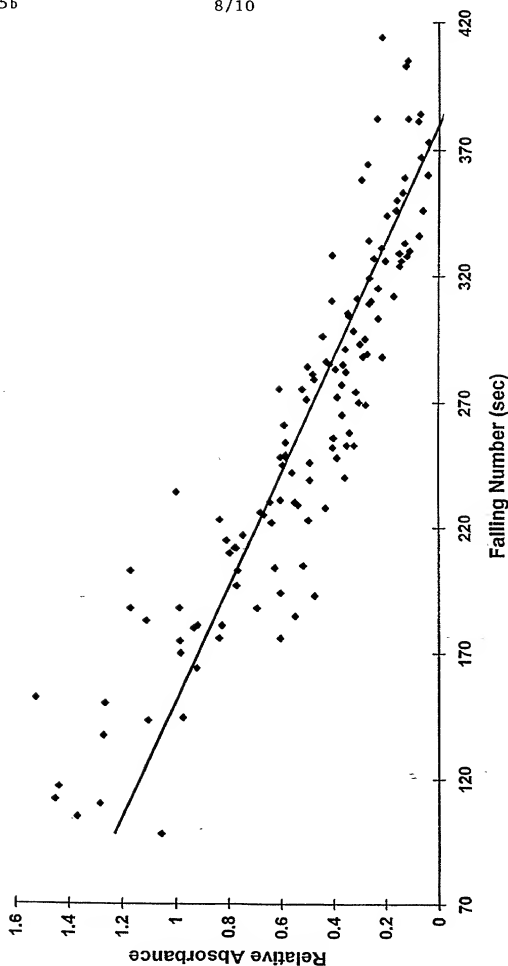
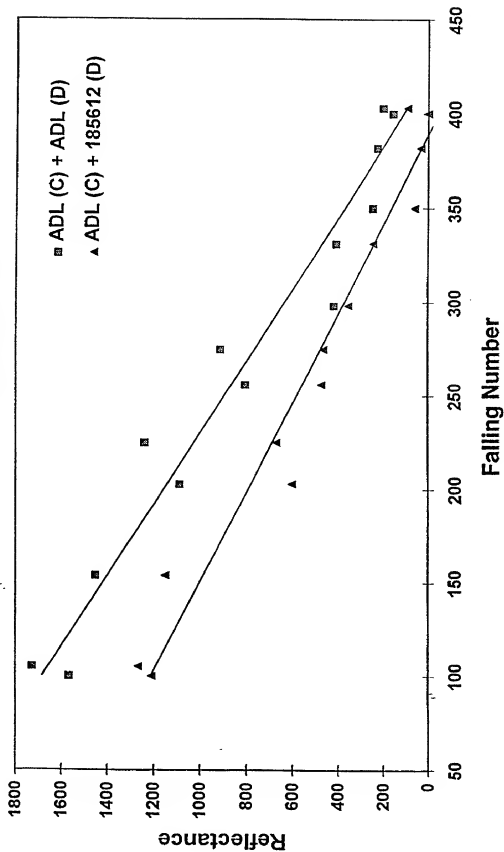




FIGURE 6

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Discrimination of sprouted and unsprouted wheat using a rapid  
immunochromatography test



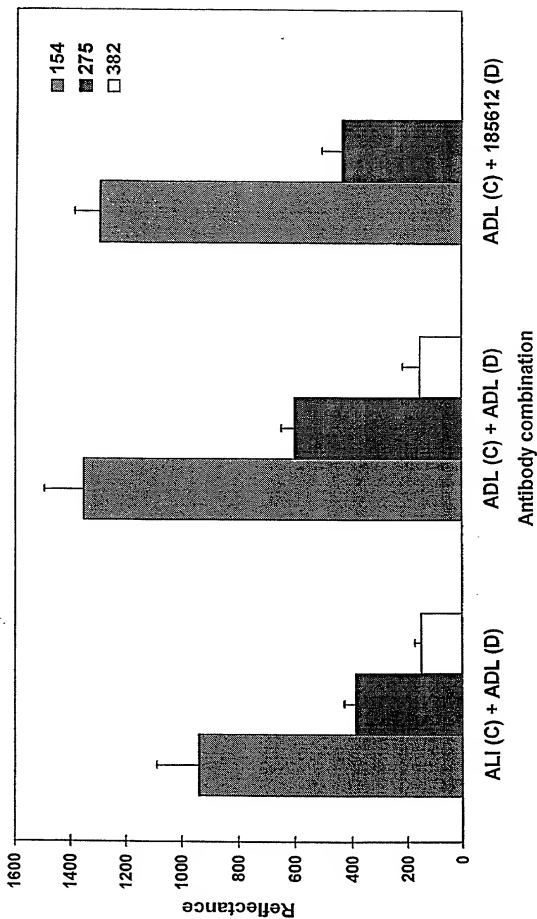
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FIGURE 7

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Within-day precision of immunochromatography (5 tests)



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Sequence Listing:

Applicant: Quality Wheat CRC Limited

Title of the Invention: Detection of preharvest sprouting in cereal grains

Number of SEQ ID NOs: 5

Software: PatentIn Ver. 2.1

SEQ ID NO: 1

Length: 15

Type: PRT

Organism: Triticum aestivum

Sequence: 1

Ile Asp Arg Leu Val Ser Ile Arg Thr Arg Gly Gln Ile His Ser

1

5

10

15

SEQ ID NO: 2

Length: 10

Type: PRT

Organism: Triticum aestivum

Sequence: 2

Cys Arg Asp Asp Arg Pro Tyr Ala Asp Gly

1

5

10

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Length: 10

Organism: *Triticum aestivum*

Val Asn Trp Val Asn Lys Val Gly Gly Ser

1                      5                      10

Length: 425

Organism: *Triticum aestivum*

Met Ala Ser Lys His Leu Ser Leu Phe Leu Val Leu Leu Gly Leu Ser

1                      5                      10                      15

Ala Ser Leu Ala Ser Gly Gln Val Leu Phe Gln Gly Phe Asn Trp Glu

20                      25                      30

Ser Trp Lys His Asn Gly Gly Trp Tyr Asn Phe Leu Met Gly Lys Val

35                      40                      45

Asp Asp Ile Ala Ala Ala Gly Val Thr His Val Trp Leu Pro Pro Ala

50                      55                      60

Ser Gln Ser Val Ser Glu Gln Gly Tyr Met Pro Gly Arg Leu Tyr Asp

65                      70                      75                      80

Leu Asp Ala Ser Lys Tyr Gly Asn Lys Ala Gln Leu Lys Ser Leu Ile

85 90 95

Gly Ala Leu His Gly Lys Gly Val Lys Ala Ile Ala Asp Ile Val Ile

100                      105                      110

Asn His Arg Thr Ala Glu Arg Lys Asp Gly Arg Gly Ile Tyr Cys Ile  
 115 120 125

Phe Glu Gly Gly Thr Pro Asp Ala Arg Leu Asp Trp Gly Pro His Met  
 130 135 140

Ile Cys Arg Asp Asp Arg Pro Tyr Ala Asp Gly Thr Gly Asn Pro Asp  
 145 150 155 160

Thr Gly Ala Asp Phe Gly Ala Ala Pro Asp Ile Asp His Leu Asn Pro  
 165 170 175

Arg Val Gln Lys Glu Leu Val Glu Leu Leu Asn Trp Leu Arg Thr Asp  
 180 185 190

Ile Gly Phe Asp Gly Trp Arg Phe Asp Phe Ala Lys Gly Tyr Ser Ala  
 195 200 205

Asp Val Ala Lys Ile Tyr Val Asp Arg Ser Glu Ala Ser Phe Ala Val  
 210 215 220

Ala Glu Ile Trp Thr Ser Leu Ala Tyr Gly Gly Asp Gly Lys Pro Asn  
 225 230 235 240

Leu Asn Gln Asp Pro His Arg Gln Glu Leu Val Asn Trp Val Asn Lys  
 245 250 255

Val Gly Gly Ser Gly Pro Gly Thr Thr Phe Asp Phe Thr Thr Lys Gly  
 260 265 270

Ile Leu Asn Val Ala Val Glu Gly Glu Leu Trp Arg Leu Arg Gly Thr  
 275 280 285

Asp Gly Lys Ala Pro Gly Met Ile Gly Trp Trp Pro Ala Lys Ala Val  
 290 295 300

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# DECLARATION, POWER OF ATTORNEY AND PETITION

As a below named inventor, I hereby declare that:

My residence, post office and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original first and joint inventor (if plural names are listed below) of the subject matter claimed and for which a patent is sought on the invention entitled: **Detection of preharvest sprouting in cereal grains**

the specification of which

☐ is attached hereto ☒ was filed on **11 November 1999** as Application Serial No. **09/830876** and was amended on **5-2&23-01** (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a)

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
PP 7058	Australia	11 November 1998	<input checked="" type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>
[Number]	[Country]	[Day/Month/Year Filed]	Yes	No

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

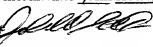
[Application Serial no]	[Filing Date]	[Status: patented, pending, abandoned]

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of the application or any patent issued thereon.

**SUGHRUE MION ZINN MacPEAK & SEAS PLLC**  
**2100 Pennsylvania Avenue NW**  
**Washington DC 20037-3202**  
**United States Of America**

with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and all future correspondence should be addressed to them.

Full name of sole or first inventor **John Howard SKERRITT**

Inventor's Signature 

Date: **14/5/01**

Residence **38 Booth Crescent, Cook, ACT, 2614, Australia** ALX

Citizenship **Australian**

Post Office Address **38 Booth Crescent, Cook, ACT, 2614, Australia**